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Efficient *in vitro* affinity maturation of phage antibodies using BIAcore guided selections

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Selection of higher affinity mutant phage antibodies has proven less than straightforward due to sequence dependent differences in phage antibody expression, toxicity to Escherichia coli, *and difficulty in eluting the highest affinity phage. These differences lead to selection for increased levels of expression or decreased toxicity rather than for higher affinity. In this work, we demonstrate how surface plasmon resonance as employed in the BIAcore can be used to increase the efficiency of phage antibody selections, yielding greater increments in affinity from a single library. A mutant phage antibody library was created by randomizing nine amino acids located in the V_L CDR3 of C6.5, a human scFv which binds the tumor antigen c-erbB-2 with a K_d of 1.6×10^{-8} M. The library was subjected to five rounds of selection in solution using decreasing concentrations of biotinylated c-erbB-2. After each round of selection, polyclonal phage were prepared and the rate of binding to c-erbB-2 determined in a BIAcore under mass transport limited conditions. Determination of the rate of binding permitted calculation of the concentration, and hence percent, of binding phage present. Results were used to select the antigen concentration for the next round of selection. To determine the optimal eluent, polyclonal phage was injected in a BIAcore and eluted using one of five different solutions (10 mM HCl, 50 mM HCl, 100 mM HCl, 100 mM triethylamine, 2.6 M $MgCl_2$). Differences were observed in eluent efficacy, which was reflected in significant differences in the affinities of phage antibodies isolated from the library after a round of selection using the different eluents. Use of the BIAcore to determine the optimal eluent and guide the antigen concentration used for selection yielded a C6.5 mutant with a 16 fold reduction in K_d ($K_d = 1.0 \times 10^{-9}$ M). This represents at least a twofold greater increment in affinity than previously obtained from a single library of phage antibodies which bind antigens.* [Hum Antibod Hybridomas 1996; 7: 97–105]

Keywords: Phage display; antibody fragment; affinity maturation; surface plasmon resonance; BIAcore; c-erbB-2

Introduction

Development of therapeutic antibodies has been limited by the immunogenicity of rodent antibodies, difficulties in adapting conventional hybridoma technology to produce human antibodies, and limits imposed on antibody affinity by the *in vivo* immune system[1]. The first two limitations have been largely overcome by the display of natural[2] and synthetic antibody variable region gene repertoires[3] on the surface of phage[4,5]. Human antibody fragments can be recovered from these libraries against virtually any antigen[2,6–9] with affinities for protein antigens ranging from 10^{-6} M to 10^{-8} M. Affinity of these primary isolates can be increased by creating mutant phage antibody libraries and selecting higher affinity antibodies[10–14].

Efficient selection of higher affinity mutant phage antibodies has proven less than straightforward due to sequence dependent differences in phage antibody expression and in toxicity to *E. coli*. These differences can lead to selection for increased expression levels, or decreased toxicity, rather than for higher affinity. In the case of single-chain Fv (scFv) phage antibodies, selection is also complicated by the tendency of some scFv to dimerize. Dimeric scFv exhibit increased apparent affinity due to avidity and are preferentially enriched over monomeric scFv when selections are performed on antigen immobilized on a solid phase[14]. Thus selections must be carefully designed to ensure enrichment based on affinity, rather than expression level, toxicity to *E. coli*, or avidity. It has been previously shown that optimal selection of higher affinity scFv phage antibodies occurs when selections are performed in solution on biotinylated antigen with subsequent capture on streptavidin-coated magnetic beads[10–14]. For the initial round of selection, an antigen concentration greater than the K_d of the wild type scFv is used in order to capture

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rare, or poorly expressed, phage antibodies. In subsequent rounds, the antigen concentration is reduced to significantly less than the desired K_d[14]. Use of too high an antigen concentration results in failure to sort on the basis of affinity, while use of too low antigen concentration results in loss of binding phage and subsequent overgrowth of deletion mutants[14]. The optimal antigen concentration cannot be predicted *a priori*, due to variability in phage antibody expression levels and uncertainty regarding the highest affinities present in the mutant phage antibody library. For selection of the highest affinity antibodies, it is also necessary to ensure that all specifically bound phage are eluted. Solutions used for elution include competition with soluble antigen[10,15,16], 100 mM triethylamine (triethylamine (TEA),[2,6,7,10,11,16], glycine, pH 2.2[17–20], 100 mM NaOAc, pH 2.8 containing 500 mM NaCl[21], or 76 mM citric acid, pH 2.8[22]. Alternatively, magnetic beads with bound phage can be added directly to *E. coli*[23].

For this work, we demonstrate how surface plasmon resonance (SPR) as employed in a BIAcore can be used to guide the antigen concentration used for selection during *in vitro* affinity maturation. Determination of the rate of binding of polyclonal phage to antigen after each round of selection permitted calculation of the concentration, and hence percent, of binding phage present. These values correlated closely with the number of positives observed by enzyme linked immunosorbent assay (ELISA). During successful phage antibody library selection, the values of bound phage remained higher after each round, in the face of decreasing antigen concentration. Reduction of the antigen concentration below a critical level led to loss of binding of polyclonal phage to antigen in the BIAcore and a loss of ELISA positive clones. Using SPR, we also demonstrated that differences exist between eluents in their ability to elute polyclonal phage antibodies from antigen. These differences led to significant differences in the affinities of antibodies selected during *in vitro* affinity maturation. Selection of the highest affinity phage antibodies required use of the proper eluent, which can be predicted using SPR.

Materials and methods

Library construction and phage preparation

A mutant phage antibody library was constructed based on the sequence of C6.5, a human scFv isolated from a nonimmune phage antibody library which binds the tumor antigen c-erbB-2 with a $K_d = 1.6 \times 10^{-8}$ M[24]. This library is described in detail in a separate publication[38]. The mutant phage antibody library C6VLCDR3 was constructed by partially randomizing nine amino acids (residues 89-95b, Kabat numbering[30]) located in the V_L CDR3 of C6.5. The ratio of nucleotides was chosen so that the frequency of wild type amino acid was 49% at each position randomized. The mutant C6.5 scFv gene repertoire was digested with SfiI and NotI and ligated into the phagemid vector pCANTAB5E (Pharmacia) digested with SfiI and NotI. After transformation, a library of 1.0×10^7 clones was obtained. For selection, phage were prepared as previously described[14].

Selection of the phage antibody library

The C6VLCDR3 library was subjected to five rounds of selection in solution on biotinylated c-erbB-2 ECD, as previously described[14], but with some modifications. After capture of phage, streptavidin-coated paramagnetic beads (Dynal) were washed a total of ten times (3 × phosphate buffered saline (25 mM NaH_2PO_4, 125 mM NaCl, pH 7.0, PBS) containing 0.05% Tween 20 (TPBS), 2 × TPBS containing 2% skimmed milk powder (MTPBS), 2 × PBS, 1 × PBS containing 2% skimmed milk powder (MPBS), and 2 × PBS) using a Dynal magnetic particle concentrator. The Dynabeads were resuspended in 1 ml PBS, and one third was used to infect 10 ml log phase *E. coli* TG1[31] which were plated on TYE plates containing 100 μg ml^{-1} ampicillin and 1% glucose (TYE-AMP-GLU)[32].

For determination of the effect of eluent, the fourth round of selection was repeated, exactly as described above, except that after washing, bound phage were eluted by adding 100 μl of one of seven eluents: 1. 100 mM HCl pH 1.0; 2. 50 mM HCl pH 1.3; 3. 10 mM HCl pH 2.0; 4. 2.6 M $MgCl_2$ 5. 100 mM TEA; 6. 1 μ c-erbB-2 ECD; or 7. no elution (magnetic beads resuspended in 1 ml of PBS). After five minutes incubation with eluent (15 min for the incubation with 1 μM c-erbB-2 ECD), the supernatant was transferred to a new tube and the mixture neutralized by the addition of 1.5 ml of 1 M Tris HCl pH 7.4. 500 μl of the elution mixture was used to infect 10 ml log phase *E. coli* TG1[31] which were plated on TYE-AMP-GLU plates.

BIAcore and ELISA screening

Phage ELISA were performed to determine the percentage of antigen binding clones. 96 single clones were picked from the unselected library and after each round of each selection, resuspended in 200 μl 2 × TY-AMP-GLU, and grown over night at 37°C in a 96-well microtitre plate (Corning). Aliquots of bacteria were transferred to a new 96-well microtitre plate containing 100 μl 2 × TY-AMP-0.1% glucose and grown to an OD_{600} of approximately 0.7. 50 μl of VCS-M13 helper phage (Stratagene) (2.5×10^8 pfu/ml) were added to each well, and the wells incubated for 1h at 37°C without shaking. 50 μl of 2 × TY-AMP containing 100 μg ml^{-1} kanamycin were added per well, and the bacteria grown overnight at 37°C. Bacteria were spun down at 2000 rpm in a Beckman GS-65 centrifuge and supernatant containing phage used for ELISA.

For phage ELISA, Immunolon 4 microtiter plates (Dynatech) were coated with 50 μl ImmunoPure avidin (Pierce; 10 μg ml^{-1} in PBS) overnight at 4°C, blocked with 1% bovine serum albumin in PBS for an hour at 37°C, incubated with 50 μl biotinylated c-erbB-2 ECD (5 μg ml^{-1} in PBS) for 30 min at 20°C, followed by an incubation with 50 μl *E. coli* supernatant containing phage for one hour at 20°C. Binding of scFv phage to the antigen was detected with a peroxidase-conjugated anti-M13 monoclonal antibody (Pharmacia) using ABTS as substrate.

The reaction was stopped after 30 min with NaF (3.2 mg ml^{-1}) and the A_{405nm} measured.

Screening of scFv by k_{off} was performed using real-time biospecific interaction analysis based on SPR in a BIAcore (Pharmacia) as described by Schier *et al.*[14]. Ten ml cultures of *E. coli* TG1 containing the appropriate phagemid was grown and expression of scFv induced with isopropylβ–D–thiogalactopyranoside (IPTG)[33]. Cultures were grown overnight at 25°C, scFv harvested from the periplasm[34], and the periplasmic fraction dialyzed for 24 h against HBS. In a BIAcore flow cell, approximately 1400 RU of c-erbB-2 ECD (25 $\mu g\ ml^{-1}$) in 10 mM acetate buffer pH 4.5 were coupled to a CM5 sensor chip using NHS/EDC amine coupling chemistry[35]. Dissociation of undiluted periplasmic fraction of *E. coli* containing scFv was measured under a constant flow of 5 $\mu l\ min^{-1}$. An apparent k_{off} was determined from the dissociation part of the sensorgram for each scFv analyzed[36].

Subcloning, expression and purification of scFv

To facilitate purification for kinetic studies, scFv genes were subcloned[24] into the expression vector pUC119 SfiNotmycHis, which results in the addition of a hexahistidine tag at the C–terminal end of the scFv. 500 ml cultures of *E. coli* TG1 harboring one of the C6.5 mutant phagemids were grown, expression of scFv induced with IPTG[33] and the culture grown at 25°C overnight. scFv was harvested from the periplasm[34], and purified by immobilized metal affinity chromatography[37] exactly as previously described[24]. To remove dimeric and aggregated scFv, samples were concentrated to a volume < 1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS. The purity of the final preparation was evaluated by assaying an aliquot by SDS–PAGE. The concentration was determined spectrophotometrically, assuming an A_{280} nm of 1.0 corresponds to an scFv concentration of 0.7 mg ml^{-1}.

Measurement of affinity and binding kinetics

The K_d of scFv were determined using SPR in a BIAcore. In a BIAcore flow cell, approximately 1400 RU of c-erbB-2 ECD (90 kDa; 25 $\mu g\ ml^{-1}$ in 10 mM sodium acetate pH 4.5) were coupled to a CM5 sensor chip[35]. Association (k_{on}) and k_{off} were measured under continuous flow of 5 $\mu l\ min^{-1}$ using a concentration range of scFv from 50 to 800 nM. k_{on} was determined from a plot of (ln(dR/dt))/t vs concentration[36]. To verify that differences in k_{on} were not due to differences in immunoreactivity, the relative concentration of functional scFv was determined using SPR in a BIAcore and was within 5% of the concentration determined by A_{280}. k_{off} was determined from the first 1.5 min of the dissociation part of the sensorgram at the highest concentration of scFv analyzed[36]. To exclude rebinding, k_{off} was determined in the presence and absence of 5.0×10^{-7} M c-erbB-2 ECD as previously described[14]. No significant differences in k_{off} were observed between samples analyzed in the presence and absence of c-erbB-2 ECD.

Determination of efficacy of eluents in removing polyclonal phage from c-erbB-2 ECD

The efficacy of different elution solutions in removing polyclonal phage from c-erbB-2 ECD was determined using SPR in a BIAcore (Pharmacia). CM5 sensor chip flow cells were coated with 1800 RU of c-erbB-2 ECD (50 $\mu g\ ml^{-1}$ in 10 mM sodium acetate pH 4.5). Polyclonal phage were prepared[14] after the third round of selection and resuspended in HBS. 30 μl of phage (5×10^{12} cfu ml^{-1} were injected over the flow cell surface using HBS as running buffer and a flow rate of 5 $\mu l\ min^{-1}$. 2.5 min into the dissociation period, 5 μl of one of six eluents (HBS, 2.6 M $MgCl_2$, 100 mM TEA, 10 mM HCl, 50 mM HCl, or 100 mM HCl) was injected over the flow cell surface at a rate of 5 $\mu l\ min^{-1}$, followed by a wash step. The amount of phage bound was determined 15 sec after the end of the association phase and six minutes later at the end of the wash step. The differences between these two points was used to calculate the percent of phage still bound after elution.

Determination of the percent of binding phage in a polyclonal phage preparation

A standard curve was constructed using monoclonal C6.5 scFv phage[25]. Phage were prepared and titred (cfu ml^{-1}) on *E. coli* TG1[14]. In a BIAcore, 1800 RU of c-erbB-2 ECD was coupled to a CM5 sensor chip using NHS-EDC chemistry. Thirty ml aliquots of C6.5 phage (1.0×10^{11} to 1.0×10^{13} cfu ml^{-1}) were injected over the flow cell surface using a running buffer of PBS containing 0.05% P20 and a flow rate of 5 $\mu l\ min^{-1}$. Binding rates were calculated in RU/min from the association portion of each sensorgram. The amount of phage bound (RU) was also determined using a reference point taken 15 sec after the end of the association phase. Two standard curves were constructed, one plotting the log of the phage titre vs the log of the binding rate, and one plotting the log of the phage titre vs the log of the RU phage bound (*Figure 1*).

To determine the concentration of binding phage in the polyclonal phage mixture, phage were prepared after each round of selection and the titre determined[14]. Thirty μl aliquots of phage (3.0 to 8.0×10^{12} cfu ml^{-1}) were injected over the flow cell surface, the binding rate and RU bound measured, and the concentration (cfu ml^{-1}) of c-erbB-2 binding phage determined from the standard curves. The percent binding phage was calculated as the ratio of the concentration of the binding phage (cfu ml^{-1})/total phage titre determined by infection of *E. coli* (cfu ml^{-1}).

Results

Monitoring phage antibody selection using surface plasmon resonance in a BIAcore

A technique was developed to measure the concentration of antigen binding phage present in a polyclonal phage mixture. To construct a standard curve relating the concentration of binding phage to BIAcore response, phage were prepared from the anti-c-erbB-2 monoclonal antibody

C6.5 [24]. The phage concentration (colony forming units (cfu) ml^{-1}) was determined by titration on *E. coli* TG1. The response signal (resonance units; RU) and rate (RU min^{-1}) of binding of serial dilutions of C6.5 scFv phage to c-erbB-2 extracellular domain (c-erbB-2 ECD) were determined in a BIAcore under mass transport limited conditions[25]. A plot of the log of the phage concentration versus either the binding rate, or the amount of phage bound yielded linear standard curves *(Figure 1)*.

To determine the utility of SPR for monitoring and guiding selections, we constructed a mutant phage antibody library C6VLCDR3 by randomizing nine amino acids located in the V_L CDR3 of C6.5, a human scFv which binds the tumor antigen c-erbB-2 with a K_d of 1.6×10^{-8} M [24]. After transformation, a library of 1.0×10^7 clones was obtained. To isolate higher affinity scFv, the library was selected on decreasing concentrations of biotinylated c-erbB-2 ECD. After each round of selection, the concentration of binding phage were determined by SPR using the standard curves shown in Figure 1 (*Table 1*). The total phage concentration (cfu ml^{-1}) was determined by titration on *E. coli* TG1 and the percentage of antigen binding phage calculated as the [binding phage (BIAcore)]/[total phage (cfu ml^{-1})]. The concentration of antigen used for the subsequent round of selection was reduced tenfold until the concentration of binding phage decreased significantly.

During the first four rounds of selection, the titre of eluted phage decreased as the antigen concentration used for selection decreased (*Table 1*). The concentration and percentage of binding phage as determined by SPR, however, increased each round (*Table 1*). The percentage of individual colonies expressing scFv which bound c-erbB-2 ECD, as determined by ELISA, also increased each round, and the values correlated closely with the percent of binding phage determined by BIAcore (*Table 1*). These results suggest successful antigen driven selection. This was confirmed by measuring the K_d of native scFv expressed from 37 clones from the fourth round of selection (see *Table 4* and the next section). All scFv had a lower K_d than the parental C6.5 scFv, with the best scFv having 16 fold decreased K_d. Further reduction of the antigen concentration to 1×10^{-12} M in a fifth round of selection resulted in a large reduction of the BIAcore response, indicating loss of binding phage antibodies due to excessive stringency (*Table 1*). Loss of phage binding in the BIAcore correlated with an absence of binding as determined by ELISA. Loss of binding was paradoxically associated with an 800 fold increase in the titre of eluted phage (*Table 1*). PCR screening of 20 clones after the fifth round of selection indicated that most clones had lost part of the scFv gene.

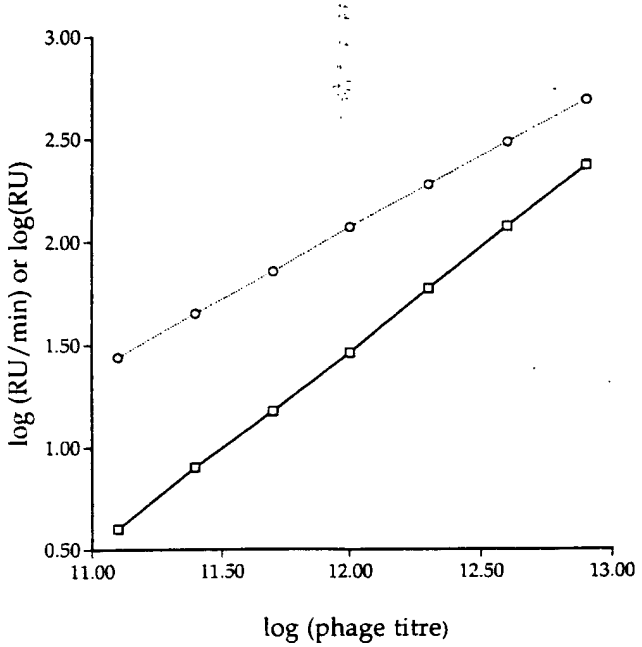

Figure 1: Correlation between phage binding in a BIAcore and the phage titre. Monoclonal C6.5 scFv (1.0×10^{11} to 1.0×10^{13} cfu ml^{-1}) were injected over a c-erbB-2 ECD coupled sensor chip in a BIAcore and the rate of binding (RU min^{-1}) and amount (RU) of phage bound determined. Two standard curves were constructed, one plotting the log of the phage concentration (cfu m^{-1}) vs the log of the binding rate (RU min^{-1}), □; and one plotting the log of the phage concentration (cfu/ml) vs the log of the amount of phage bound (RU), ○. These standard curves were used to calculate the titre of binding phage in polyclonal mixtures of phage

Table 1: Results of selection of the C6VLCDR3 phage antibody library on c-erbB-2 ECD

Round of selection	Antigen conc. used for selection [$\times 10^{-9}$ M]	Titre of eluted phage [phage/ml]	Phage binding by ELISA (%)	Titre of phage preparation [$\times 10^{12}$ phage/ml]	BIAcore response [RU]	BIAcore phage titre [$\times 10^{12}$ phage/ml]	BIAcore phage binding (%)
0	—	1.0×10^7	0	3.0	41	0.2	6
1	40	1.5×10^5	11	11.0	70	0.5	5
2	1	4.0×10^4	73	2.5	159	2.0	80
3	0.1	2.0×10^4	86	2.0	191	1.6	80
4	0.01	1.0×10^4	100	1.7	227	1.6	94
5	0.001	8.0×10^6	3	5.0	49	0.3	6

A phage antibody library consisting of V_L CDR3 mutants of C6.5 scFv was subjected to five rounds of selection using decreasing antigen concentration (column 2). After each round of selection, the titre of eluted phage (column 3) was measured, the percent of individual clones binding antigen (column 4) was determined by ELISA, and the polyclonal phage was prepared and titred for the next round of selection (column 5). The amount (RU) of polyclonal phage binding to a c-erbB-2 ECD coupled sensor chip was measured in a BIAcore (column 6) and used to determine a titre of binding phage (column 7) from a standard curve constructed using known concentrations of the monoclonal phage antibody C6.5 (see *Figure 1*). The percent of binding phage calculated by BIAcore (column 8 = (column 7/column 6)) correlated closely with the percentage of individual clones binding c-erbB-2 ECD by ELISA (column 4).

BIAcore analysis and optimization of elution conditions for antibody phage selection

To determine if differences existed in the ability of eluents to remove antigen bound phage, polyclonal phage were prepared after three rounds of selection of the C6VLCDR3 library and studied using SPR in a BIAcore. After an initial bulk refractive index change, binding of phage to immobilized c-erbB-2 ECD was observed, resulting in an average of 189 RU bound (*Table 2*, *Figure 2*). Phage were then allowed to either spontaneously dissociate from c-erbB-2 ECD using hepes buffered saline (HBS) as running buffer, or were eluted with either 100 mM HCl, 50 mM HCl, 10 mM HCl, 2.6 M $MgCl_2$, or 100 mM TEA. Major differences were observed between eluents in their ability to remove bound phage (*Table 2*, *Figure 2*). The most effective solutions in removing bound phage antibodies were 100 mM HCl and 50 mM HCl, followed by 100 mM TEA. 2.6 M $MgCl_2$ (which removes 100% of wild type C6.5) and 10 mM HCl were only minimally more effective than the running buffer in removing bound phage.

Table 2: Effects of different eluents on removing bound phage from c-erbB-2 ECD as determined by surface plasmon resonance in a BIAcore

Eluent	RU phage bound before elution	RU phage bound after elution	% bound phage eluted
Hepes buffered saline	190	150	21
2.6 M $MgCl_2$	192	141	27
100 mM triethylamine	195	84	57
10 mM HCl	189	127	33
50 mM HCl	182	0	100
100 mM HCl	185	0	100

Polyclonal anti-c-erbB-2 phage prepared after the third round of selection were injected over c-erbB-2 ECD coupled to a sensor chip in a BIAcore. After association, the amount (RU) of bound phage was determined, one of six eluents injected over the sensor chip surface, and the amount of phage that remained bound to c-erbB-2 determined. Major differences were observed in the efficacy of eluents in removing bound phage.

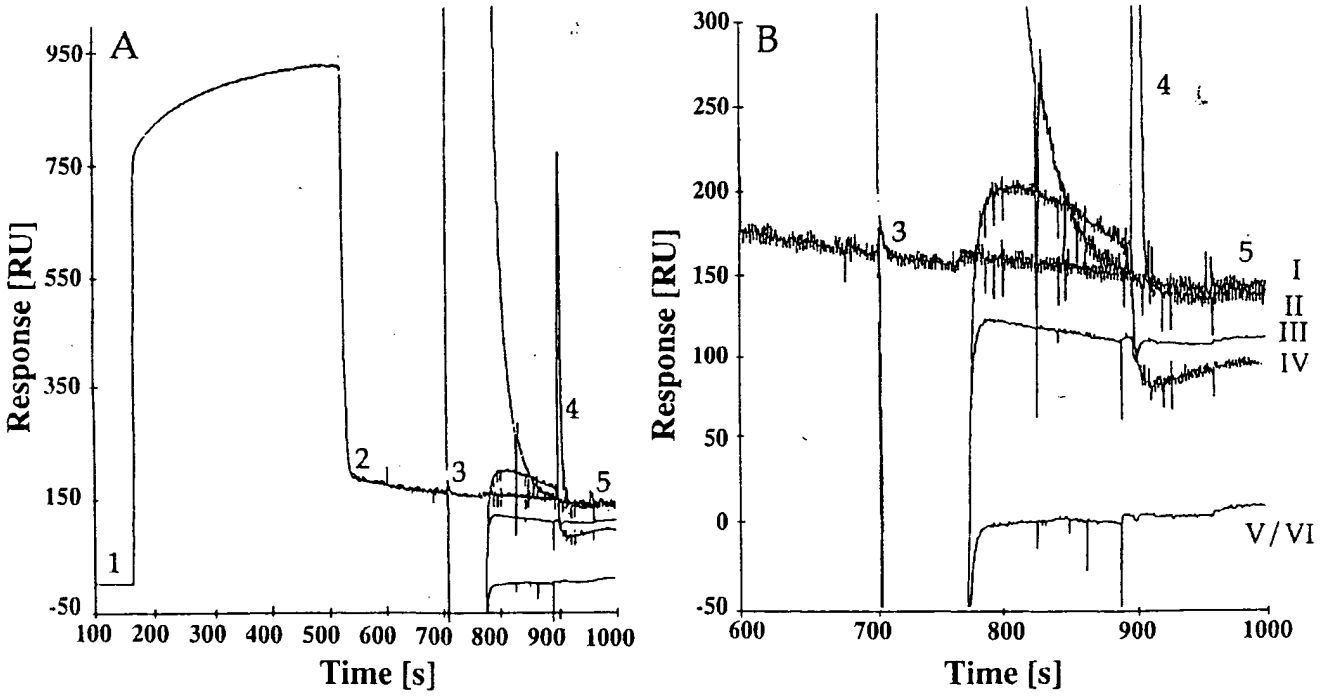

Figure 2: Effect of different eluents on removing bound phage from c-erbB-2 ECD. Polyclonal anti-c-erbB-2 phage were injected over a c-erbB-2 ECD coupled sensor chip in the BIAcore and the ability of six different eluents to remove bound phage was determined. **A:** overlay plot of the six sensorgrams generated from analysis of the six eluents: 1. baseline, beginning of association; 2. beginning of dissociation; difference between point 1 and 2 = amount of phage bound; 3. beginning of elution, differences in refractive index of eluents results in large positive or negative changes in RU, depending on the eluent used; 4. washing out the eluent from the flowcell; 5. amount of phage bound after elution. **B:** Enlargement of (A) between points 3 and 5. Significant differences exist in the amount of phage remaining bound after elution. (I : HBS; II : 2.6 M $MgCl_2$; III : 10 mM HCl; IV : 100 mM TEA; V : 50 mM HCl; VI : 100 mM HCl)

Table 3: Effect of different eluents on the selection of higher affinity phage antibodies

Eluent	Titre of eluted phage	ELISA positive clones	K_d ($\times 10^{-9}$ M)	k_{on} ($\times 10^5$ $s^{-1}M^{-1}$)	k_{off} ($\times 10^{-3}$ s^{-1})
No elution[a]	5.2×10^4	75/92	5.39 ± 0.73	4.64 ± 0.35	2.49 ± 0.41
1 μM c-erbB-2	6.0×10^4	82/92	5.99 ± 1.12	5.09 ± 0.27	2.58 ± 0.47
2.6 M $MgCl_2$	1.1×10^4	83/92	3.30 ± 0.45[c]	5.05 ± 0.43	1.58 ± 0.14
100 mM TEA	1.2×10^4	89/92	2.65 ± 0.35[b]	4.78 ± 0.39	1.27 ± 0.20
10 mM HCl	1.0×10^4	85/92	6.09 ± 1.29	5.72 ± 0.30	3.46 ± 0.80
50 mM HCl	1.0×10^4	90/92	2.60 ± 0.40[b]	6.38 ± 1.02	1.54 ± 0.19
100 mM HCl	2.1×10^3	87/92	2.52 ± 0.46[b]	5.99 ± 0.37	1.40 ± 0.20

Polyclonal phage was subjected to a fourth round of selection on c-erbB-2 ECD and the bound phage eluted with one of seven eluents (column 1). The titre of eluted phage (column 2) and the number of individual clones binding c-erbB-2 was determined by ELISA. scFv with the lowest k_{off} were identified by BIAcore screening, the scFv purified, and binding kinetics (k_{on} and k_{off}) determined by BIAcore and used to calculate the K_d. Significant differences in the K_d of selected scFv were observed.
[a], magnetic beads with bound phage added directly to *E. coli* culture; [b] $p < 0.05$ compared to no elution, 1 μM c-erbB-2, and 10 mM HCl; [c] $p < 0.05$ compared to 10 mM HCl.

Table 4: Effect of elution solutions on the sequences, affinities and binding kinetics of purified scFv

Clone	V_L CDR3 sequence	K_d ($\times 10^{-9}$ M)	k_{on} ($\times 10^5$ s^{-1} M^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})
C6.5	A A W D D S L S G W V	16.0	4.0 ± 0.20	6.3 ± 0.06
No elution				
C6ML3-5(4)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-17	- S - - Y Y R - - - -	4.9	3.5 ± 0.22	1.7 ± 0.02
C6ML3-1	- - - - Y - - W - - -	6.1	3.3 ± 0.07	2.0 ± 0.15
C6ML3-22	- - - - A - - - - - -	8.3	4.3 ± 0.21	3.6 ± 0.02
C6ML3-26	- - - - - - R - - - -	8.3	6.0 ± 0.77	5.0 ± 0.04
1 μM c-erbB-2				
C6ML3-5(5)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-17	- S - - Y Y R - - - -	5.0	3.5 ± 0.22	1.7 ± 0.02
C6ML3-25(2)	- - - - N R H - - - -	7.4	5.9 ± 0.72	4.4 ± 0.03
2.6 M $MgCl_2$				
C6ML3-12	- - - - Y - R - - - -	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-15	- - - - R P - W - - -	2.2	5.9 ± 0.8	1.3 ± 0.02
C6ML3-7(2)	- - - - Y A V - - - -	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-5(2)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-16	- S - - Y - R - - - -	3.8	5.5 ± 0.12	2.1 ± 0.05
C6ML3-17	- S - - Y Y R - - - -	4.9	3.5 ± 0.22	1.7 ± 0.02
100 mM TEA				
C6ML3-19	- S - - R P - W - - -	1.5	6.6 ± 0.69	1.0 ± 0.02
C6ML3-12	- - - - Y - R - - - -	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-18	- S - - A - - W - - -	2.4	2.6 ± 0.08	0.62 ± 0.02
C6ML3-20	- - - E Q - - W - - -	3.0	4.7 ± 0.08	1.4 ± 0.02
C6ML3-5(3)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
10 mM HCl				
C6ML3-23	- S - - H - - W - - -	1.5	6.7 ± 0.41	1.0 ± 0.02
C6ML3-7	- - - - Y A V - - - -	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-5	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-21	- - - - Y - Q - - - -	4.5	4.9 ± 0.01	2.2 ± 0.05
C6ML3-25	- - - - N R H - - - -	7.4	5.9 ± 0.72	4.4 ± 0.03
C6ML3-22	- - - - A - - - - - -	8.3	4.3 ± 0.21	3.6 ± 0.02
C6ML3-26	- - - - - - R - - - -	8.3	6.0 ± 0.77	5.0 ± 0.04
C6ML3-24	- - - - E Q I F - - -	12.4	6.4 ± 0.89	7.9 ± 0.04
50 mM HCl				
C6ML3-12(2)	- - - - Y - R - - - -	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-29	- - - - G T - W - - -	1.7	12.9 ± 1.03	2.2 ± 0.02
C6ML3-28	- S - - Y A - - - - -	2.5	6.8 ± 0.17	1.7 ± 0.02
C6ML3-7(2)	- - - - Y A V - - - -	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-6	- S - - Y - - - - - -	3.2	5.9 ± 0.43	1.9 ± 0.02
C6ML3-17	- S - - Y Y R - - - -	4.9	3.4 ± 0.22	1.7 ± 0.02
100 mM HCl				
C6ML3-9	- S - - Y T - - - - -	1.0	7.6 ± 0.20	0.76 ± 0.03
C6ML3-14(2)	- - - - - P - W - - -	1.1	7.0 ± 0.40	0.77 ± 0.02
C6ML3-15	- - - - R P - W - - -	2.2	5.9 ± 0.80	1.3 ± 0.02
C6ML3-5(4)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09

To determine if differences observed in the BIAcore were reflected in the affinity of selected scFv, a fourth round of selection was performed on the C6VLCDR3 phage antibody library. Phage were prepared from the third round of selection and elutions were performed using one of seven regimens: 1. 100 mM HCl; 2. 50 mM HCl; 3. 10 mM HCl; 4. 2.6 M $MgCl_2$; 5. 100 mM TEA; 6. 1 μM c-erbB-2 ECD; and 7. no elution (magnetic beads resuspended in 1.5 ml 1 M Tris HCl pH 7.4). After the fourth round of selection, only minor differences were observed in the frequency of ELISA positive scFv (*Table 3*). The titre of eluted phage, however, was 6 to 30 times lower when elutions were performed with $MgCl_2$, HCl, or TEA, compared to not eluting, or eluting with antigen (*Table 3*). To screen for the highest affinity scFv, native scFv was expressed from 24 ELISA positive clones and the dissociation rate constant (k_{off}) determined without purification. scFv was purified from the eight clones with the lowest k_{off} from each of the seven elution regimens, the k_d, k_{on}, and k_{off} determined, and the scFv gene sequenced (*Table 4*). scFv resulting in elutions with 50 mM HCl, 100 mM HCl, and 100 mM TEA had significantly lower K_d than scFv resulting from elutions with 10 mM HCl, 1 μM c-erbB-2 ECD, or no elution (*Table 3*, *Table 4*). Elution with 100 mM HCl resulted in selection of the two highest affinity clones (*Table 4*, C6ML3-9 and C6ML3-14), however the difference in average affinity between elution with 100 mM HCl, 50 mM HCl, or 100 mM TEA was not statistically significant (*Table 3*). The different eluents, however, did yield scFv with similar kinetic properties but different sequences in V_L CDR3 (*Table 4*).

Discussion

Phage display has proven to be a powerful tool for increasing antibody affinity[10–14,16]. To make the process efficient, however, it is essential to obtain the highest affinity clones from each mutant library. This is not necessarily straightforward, since enrichment ratios depend not only on affinity, but also on differences in expression level, folding efficiency, and toxicity to *E. coli*. Selection on the basis of affinity is optimal when selections are performed in solution and the antigen concentration is reduced each round[14]. Failure to adequately reduce the antigen concentration results in failure to sort on the basis of affinity, while too large a reduction results in loss of binding phage[14]. Moreover, the optimal antigen concentration cannot be predicted *a priori*, due to variability in phage antibody expression levels and uncertainty regarding the highest affinities present in the mutant phage antibody library. Our data indicate that the stringency of selections can be monitored in a BIAcore by measuring the concentration and percentage of binding phage present in polyclonal phage prepared after each round of selection. The results can then be used to determine the antigen concentration used for the next round of selection. As in our example, little or no change in the binding phage concentration indicates that the antigen concentration can be decreased significantly (at least tenfold) in the next round of selection. A rapid drop in binding phage between rounds suggests either the need to repeat the round using a higher antigen concentration, or a conservative change in the antigen concentration used for the subsequent round.

As an alternative to monitoring selection using BIAcore, the titre of eluted phage is frequently followed, a rising titre being indicative of positive selection[10,18,23]. In this and previous work[14,26], however, we observed that positive selection on the basis of affinity occurred despite falling titres of eluted phage. When the titre of eluted phage did increase, there was a loss of binding phage, as determined by both BIAcore and ELISA. The mechanism for the increase in titre is unclear, however the majority of these phage have deleted at least one portion of the scFv gene. This could result in increased infection efficiency, due to the greater number of wild type pIII on the phage surface, or reduced toxicity to *E. coli* from leaky scFv expression. Regardless, following the titre of eluted phage is not a useful monitor of selection.

One potential limitation of this technique is that the concentration of binding phage is also affected by the efficiency with which mutant scFv are expressed on phage relative to the expression level of the phage antibody used to construct the standard curve. This would be reflected in a greater difference between the number of binding phage determined by ELISA compared to the value determined by BIAcore. In selected other C6.5 based libraries, we have observed differences as great as 1.5 fold between percentages of positive binders determined by BIAcore and ELISA. In these instances, the expression levels of native scFv was also significantly lower than the expression level of C6.5 (RS and JDM, unpublished data).

Our results also demonstrate the important effect of eluent choice on the affinities of selected antibodies, even when using limiting antigen concentration and BIAcore screening to identify the highest affinity scFv. Two previously described elution regimens were found to be the least effective for selecting higher affinity antibodies; infecting without elution by adding magnetic beads with antigen-bound phage directly to *E. coli* cultures[23], and competitive elution of scFv with soluble antigen[10,15,16]. When eluting by incubating phage bound to antigen with *E. coli*, the phage probably must dissociate from antigen for infection to occur. Steric hindrance, due to the size of paramagnetic beads, blocks the attachment of pIII on antigen bound phage to the f-pilus on *E. coli*. This would result in preferential selection of scFv with rapid k_{off}, consistent with our results. Since a reduction in k_{off} is the major mechanism for decreases in K_d, this results in the selection of lower affinity scFv. Eluting with soluble antigen has a similar effect of the kinetics of selected scFv. The phage must first dissociate from immobilized antigen, then rebinding is blocked by binding of the phage to soluble antigen. Phage antibodies with the lowest k_{off} will remain bound to immobilized antigen and therefore are not available for infection of *E. coli*.

The optimal type of eluent (acidic, basic, chaotropic) and concentration required will depend on the phage antibody affinity[27,28] and the type of bonds that need to be interrupted. This will vary considerably between libraries, depending on the nature of the antigen–antibody interaction. In this example, significantly higher affinity scFv were obtained eluting with HCl, pH 1.3 compared to HCl, pH

2.0. In fact, the affinities of scFv isolated after elution with HCl, pH 2.0 were no different than results obtained without eluting. Similarly, we studied 2.6 M $MgCl_2$, because we had previously determined it would remove 100% of bound wild type C6.5[24]. This concentration of $MgCl_2$, however, was ineffective in eluting C6.5 V_L CDR3 mutants. Eluting with higher concentrations of $MgCl_2$ would have resulted in the selection of higher affinity scFv. For example, 3 M $MgCl_2$ was required to elute 100% of C6L1 scFv ($K_d = 2.5 \times 10^{-9}$M)[14] from a c-erbB-2 ECD BIAcore sensor chip and 4 M $MgCl_2$ was required to elute 100% of C6ML3-9 ($K_d = 1.0 \times 10^{-9}$M).

A convenient way of predicting the optimal eluent is to analyze polyclonal phage in a BIAcore. The results can then be used to design elution conditions to achieve optimal enrichment for higher affinity clones. One approach would be to elute sequentially, using a less stringent eluent to remove low affinity binders, followed by a more stringent eluent to remove high affinity binders. Thus the BIAcore information is used to select 'washing' reagents which remove lower affinity phage antibodies more effectively than PBS. This could reduce the number of selection rounds and amount of screening required to select and identify the highest affinity binders. This strategy might also be useful to isolate antibodies to low density antigens on intact cells or tissue. A mild eluent could be used to remove low affinity phage antibodies, which are preferentially selected due to high density antigen present on the cell surface, as well as nonspecifically bound phage. Phage specific for lower density antigens would then be removed using a more stringent solution.

An alternative to eluting with stringent solutions is to use antigen biotinylated with NHS-SS-Biotin (Pierce)[7]. All of the bound phage can be released from the magnetic beads by reducing the disulfide bond between antigen and biotin. One advantage of this approach is that elution of all phage is guaranteed. Use of NHS-SS-Biotin could be combined with use of a milder eluent for washing (determined by BIAcore analysis) to increase enrichment for higher affinity phage antibodies. Our results suggest, however, that use of stringent eluents that are chemically different (acidic, basic, or chaotropic) results in the selection of scFv of equally high affinity, but of different sequence. Isolation of scFv of different sequences has a number of advantages. Single amino acid changes can affect expression levels in *E. coli* dramatically[29]. For example, expression level of C6ML3-5 (100 $\mu g\,l^{-1}$) was 100 times less than for wild type C6.5 (10 mg l^{-1}). Furthermore, different scFv might have different physicochemical characteristics (dimerization, stability, or immunoreactivity) or even different effects *in vivo* (specificity, biodistribution, or clearance). Thus parallel selections using different stringent eluents should result in a greater number of high affinity binders than use of a single eluent.

The K_d of C6.5 was decreased 16 fold from a single library of V_L CDR3 mutants. This is at least twofold greater than the three to eightfold decreases in K_d previously obtained from a single library for protein binding phage antibodies. We conclude that this greater efficiency in affinity maturation results from use of the BIAcore to monitor and guide selections, and use of the optimal eluent, rather than the specific CDR selected for mutagenesis[12,13]. For example, using BIAcore guidance, the K_d of C6ML3-9 was reduced an additional ninefold by randomizing four amino acids in V_H CDR3[38]. Use of the BIAcore to guide selections should decrease the number of libraries required to achieve the desired K_d.

Nomenclature

AMP	ampicillin
CDR	complementarity determining region
c-erbB-2 ECD	extracellular domain of c-erbB-2
cfu	colony forming units
ELISA	enzyme linked immunosorbent assay
GLU	glucose
HBS	hepes buffered saline, 10 mM hepes, 150 mM NaCl, pH 7.4
IPTG	isopropylβ–D–thiogalactopyranoside
k_{on}	association rate constant
k_{off}	dissociation rate constant
MPBS	2% skimmed milk powder in PBS
MTBS	2% skimmed milk powder in TPBS
PBS	phosphate buffered saline, 25 mM NaH_2PO_4, 125 mM NaCl, pH 7.0
RU	resonance units
scFv	single-chain Fv fragment
SPR	surface plasmon resonance
TEA	triethylamine
TPBS	0.05% v/v Tween 20 in PBS
V_H	immunoglobulin heavy chain variable region
V_L	immunoglobulin light chain variable region

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